44 Stone, J.M. *et al.* (1998) Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* 117, 1217–1225
45 Stone, J.M. *et al.* (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine/threonine receptor kinase. *Science* 266, 793–795
46 Braun, D.M. *et al.* (1997) Interaction of the maize and *Arabidopsis* kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signaling in plants. *Plant J.* 12, 83–95
47 Yamamuro, C. *et al.* (2000) Loss of function of a rice brassinosteroid insensitive1 homolog prevents internode elongation and bending of the lamina joint. *Plant Cell* 12, 1591–1605
48 Mendoza, C. *et al.* (1995) Nongenomic steroid action: independent targeting of a plasma membrane calcium channel and a tyrosine kinase. *Biochem. Biophys. Res. Commun.* 210, 518–523
49 Migliaccio, A. *et al.* (1996) Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol–receptor complex in MCF-7 cells. *EMBO J.* 15, 1292–1300
50 Klahre, U. *et al.* (1998) The *Arabidopsis DIMINUTO/DWARF1* gene encodes a protein involved in steroid synthesis. *Plant Cell* 10, 1677–1690
51 Szekeres, M. *et al.* (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in *Arabidopsis*. *Cell* 85, 171–182
52 Koncz, C. (1998) Crosstalk between brassinosteroids and pathogenic signalling? *Trends Plant Sci.* 3, 1–2

Experimental mouse genetics – answering fundamental questions about mammary gland biology

Jonathan M. Shillingford and Lothar Hennighausen

Contemporary gene-targeting techniques now make it possible to alter specific genes in the genome. As a result, a plethora of mouse models have been generated that allow researchers to dissect cell-signaling pathways involved in mammary gland development and in breast cancer. But what have we learned so far? What do these models teach us? This review presents a global picture of how the analyses and comparison of individual knockout mouse models provide important insights into basic mammary gland biology. Particular emphasis is placed upon what is currently known about the signaling pathways involved in the establishment of the mammary ductal tree, and its subsequent proliferation at pregnancy and lactation. In addition to these well-established pathways, we address recent data that describe the role of lesser-known genes in the development of the mammary epithelium.

Jonathan M. Shillingford*
Lothar Hennighausen
Laboratory of Genetics and Physiology, National Institutes of Health, 9000 Rockville Pike, Bethesda, MD 20817, USA.
*e-mail: jonshi@helix.nih.gov

Our knowledge of mammary gland biology has benefited greatly in the past decade from the development of modern mouse genetics. These tools enable us to target specific genes and to analyze the impact of their loss on cell fate and function. With these approaches, several receptors, ligands and transcription factors (Table 1) have been linked to mammary gland development and function. Although it is clear from gross morphology that these models exhibit defects in mammopoiesis, relatively little is known about the molecular events and signaling pathways that contribute to the observed phenotypes. An understanding of the individual signaling components, their actions and their ability to crosstalk with each other is necessary to ascribe a functional role to these pathways during normal mammary development. In addition, knowledge of transcription-factor target genes is desirable so that we can begin to define development on a genetic level. In this article, we present a broad overview of our current understanding of how signaling pathways contribute to mammopoiesis. Furthermore, we highlight the importance of mammary stromal–epithelial interactions and the discovery of new genes involved in mammary gland development.

A compartmental analysis of signaling pathways

The mammary gland consists of several cell types and the ratio of these cell types changes during development (Fig. 1). In the virgin, adipocytes predominate as the ductal epithelium invades the fat pad (Fig. 1a,b). During pregnancy (Fig. 1c,d), the epithelium proliferates so that at lactation (Fig. 1e) the secretory epithelium completely fills the fat pad. Once the gland is no longer suckled, the epithelium undergoes apoptosis and reverts back to a virgin-like state (Fig. 1f). A variety of steroid hormones, cytokines, growth factors and the signaling pathways that these molecules activate control the rapid changes that occur during these developmental phases. To complicate issues further, there is communication between the stromal compartment and the epithelial compartment (reviewed in Refs 1,2). Although these features make the mammary gland an interesting tissue to study, they can make interpretations complicated at best.

Studies have been performed to address these compartmentalization issues by utilizing receptor knockout mice and the technique of mammary tissue transplantation[3–6]. This transplantation procedure was first described in 1959 (Ref. 7) and has seen a revival in recent years. As depicted in Fig. 2, a variety of transplants is possible. The most common transplants are mutant epithelium into wild-type stroma (Fig. 2b) and wild-type epithelium into mutant stroma (Fig. 2a). Transplanting mutant

Table 1. Receptor-, ligand- and transcription factor-deficient mouse-models that exhibit mammary gland phenotypes[a]

Receptors and ligands	Refs	Transcription factors	Refs
Pgr	9,16	Stat5a	31
Pgr-A (overexpression)	21	Stat5a/b	32
Pgr-A (deletion)	19	Stat3	46
Esr1	8,13,20	Cebpb	47–49
Esr2	18	Idb2	36
Egfr	5,50,51	Foxb1	52
Tgfbr2	53	Mybl1	54
Ghr	6,55		
Prlr/Prl	6,15,22,56		
Inhbb	4		
RANK/RANKL	35		
Ddr1	37		
Csf1	57		
Oxytocin	58,59		

[a]Abbreviations: Cebpb, CCAAT/enhancer binding protein β; Csf1, colony stimulating factor 1; Ddr1, discoidin domain receptor 1; Egfr, epidermal growth factor receptor; Esr1, estrogen receptor α; Esr2, estrogen receptor β; Foxb1, forkhead box B1; Ghr, growth hormone receptor; Idb2, inhibitor of differentiation and DNA-binding 2; Inhbb, inhibin β-B; Mybl1, myeloblastosis oncogene-like 1; Pgr; progesterone receptor; Prl, prolactin; Prlr, prolactin receptor; RANK, receptor activator of NFκB; RANKL, receptor activator of NFκB-ligand; Stat, signal transducer and activator of transcription; Tgfbr2, transforming growth factor β receptor II.

epithelium into wild-type stroma can address possible indirect effects on mammary gland development that occur as a result of the overexpression or inactivation of functional genes in cell types outside the mammary gland (e.g. changes that cause an increase or a decrease in systemic hormone levels). In turn, transplanting wild-type epithelium into mutant stroma assesses whether functional alteration of specific genes affects communication and/or interactions between the stromal and the epithelial compartments. Other possible combinations of transplants are shown for completeness (Fig. 2). More recently, another mammary transplantation approach was developed where tissue recombinants are transplanted under the renal capsule[8].

The first study that examined the role of stromal–epithelial interactions using mammary tissue transplantation utilized mice deficient in inhibin β-B (Inhbb), a member of the transforming growth factor-β (Tgfb) family[4]. It was shown that, although intact wild-type epithelium and stroma developed normally in *Inhbb*-null hosts (Fig. 2e), *Inhbb*-null epithelium and stroma remained underdeveloped in wild-type hosts (Fig. 2f). Transplantation of *Inhbb*-null epithelium into the cleared fat pad of wild-type hosts (Fig. 2a) resulted in normal development. This provided evidence that the absence of Inhbb in the stroma was responsible for impaired mammary development. Similarly, epidermal growth factor receptor (Egfr) is required only in the stromal compartment and its absence in the epithelial compartment does not perturb mammary gland development[5,6]. By contrast, the progesterone receptor (Pgr) is required in both the epithelial and stromal compartment for successful branching of the ductal epithelium[9].

These studies demonstrate that signaling between the stromal and epithelial compartments are necessary to form a functional mammary gland (reviewed in Ref. 10). Although it is not fully understood how such signaling mechanisms elicit their effects, it is possible that partitioning of these signaling cascades in different compartments contribute to the spatial–temporal dynamics of mammary gland development. For example, stromal signals that act on the epithelium could provide biomolecular cues that help determine branching morphogenesis[11], the extent of ductal outgrowth and the position of ducts within the fat pad. Conversely, signals emanating from the epithelial compartment that act on stromal cells might serve to mobilize fat stores within adipocytes that support proliferation and recruit other cell types necessary for ductal infiltration (e.g. macrophages and eosinophils[12]). Although these signals are partitioned, this does not mean they are mutually exclusive.

The signals and genes that specify the mammary epithelial cell

The combined actions and interactions of steroid hormones, cytokines and growth factors, their respective signaling pathways and intercommunication between the stromal and epithelial compartments are all responsible for the growth and regression of the mammary gland throughout its development. To this end, we propose that programmatic cellular responses exist that help to define the distinct changes that take place in mammary tissue. Such changes include the development of ductal branch points, determination of ductal and secretory epithelial cells, the organization of the mammary gland into functional alveolar structures capable of coordinated milk secretion and finally, at involution, the regression of the gland back to a virgin-like state.

It has been demonstrated that estrogen, progesterone and prolactin are involved in the process of ductal elongation, ductal side branching and alveolar development, respectively[13–15]. These observations have been supported by functional genetic studies using mice deficient in genes encoding the estrogen receptor (*Esr1* and *Esr2*), progesterone receptor (*Pgr*) or prolactin receptor (*Prlr*)[13,16–20]. The *Esr1*-null mice show multiple defects in the female reproductive system[13], which, in turn, is required for mammary ductal development and outgrowth during puberty. To avoid possible indirect effects on mammary gland development that result from altered levels of circulating hormones, transplantation and hormonal replacement studies were performed[8,20]. These experiments revealed that, in the absence of Esr1 only, a rudimentary ductal system develops. Further experiments using tissue recombinants demonstrated that ductal development

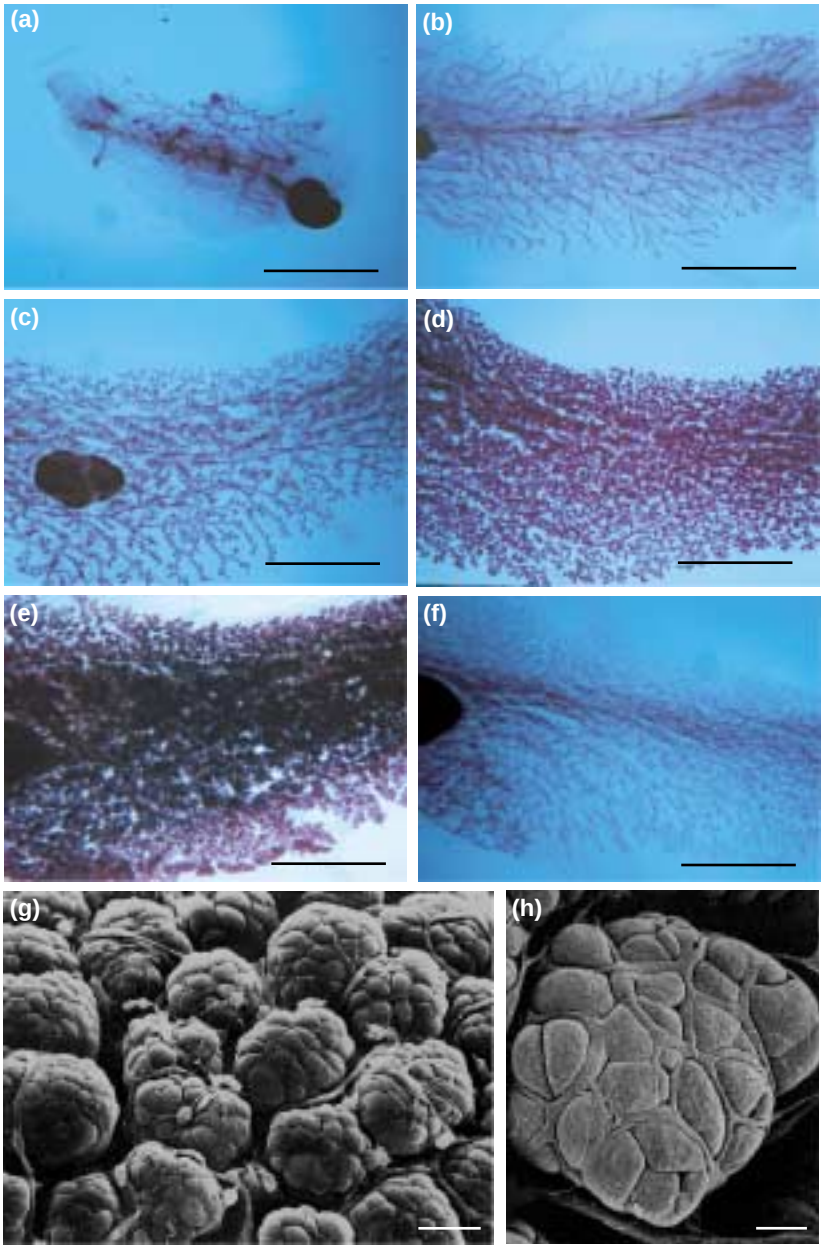

Fig. 1. Development and structural architecture of the mammary gland. (a–f) show the development of the mammary epithelium at (a) three weeks, (b) ten weeks, (c) pregnancy day 11, (d) pregnancy day 16, (e) lactation day one and (f) involution day ten. (g) depicts a scanning electron micrograph (SEM) of the grape-like structure of a group of alveoli. (h) is a scanning electron micrograph of a single alveolus, which is shown to be composed of secretory cells surrounded by a basketwork of myoepithlelial cells. Scale bars (a–f) = 1 mm; (g) = 20 μm; (h) = 10 μm. SEMs in (g) and (h) reproduced, with permission, from Ref. 60. © Springer-Verlag.

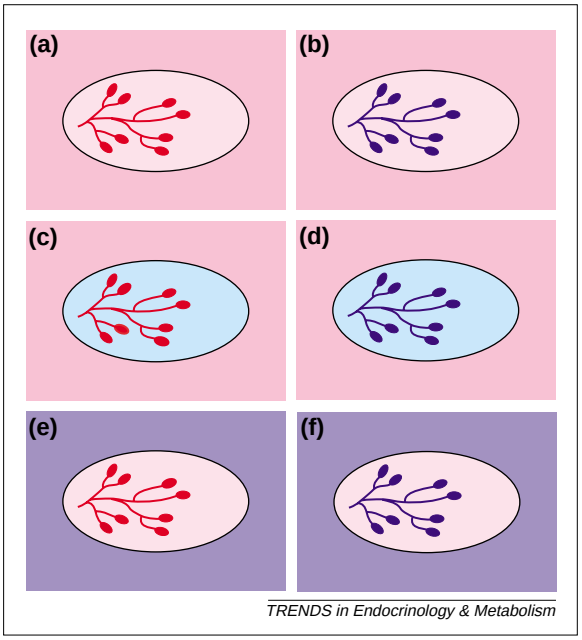

Fig. 2. Permutations of the mammary transplantation technique. (a–f) show possible combinations of mammary transplantation. The most common combination is depicted in (b) (mutant epithelium transplanted into the stroma of a wild-type mouse). Red, wild-type epithelium; dark blue, mutant epithelium; light pink, wild-type stroma; light blue, mutant stroma; dark pink, wild-type mouse; purple, mutant mouse.

requires Esr1 in the stromal compartment, but not in the epithelial compartment[8]. By contrast, targeted disruption of *Esr2* had no apparent effect on the mammary gland at any stage of development[18], highlighting the overall importance of Esr1 in mediating estradiol action during ductal growth.

Disruption of *Pgr* results in the development of a normal mammary ductal tree but a lack of ductal side branches[9,16]. Because it is possible that Pgr-A and/or Pgr-B are responsible for the phenotype observed, the relative contribution of the two forms of the receptor was recently assessed[19,21]. One study examined the introduction of an additional Pgr-A transgene in an attempt to shift the relative synthesis of the Pgr-A and Pgr-B isoforms[21]. Mammary glands isolated from ovariectomized prepubertal Pgr-A transgenic mice showed persistent terminal end buds (TEBs) with abnormal development and morphology. Furthermore, adult *Pgr-A*-transgenic mice showed defects in basement membrane integrity and cell–cell adhesion. In another study, the *Pgr-A* gene was selectively disrupted, leaving the *Pgr-B* gene intact[19]. Analyses of the mammary glands in these mice revealed that they responded normally to estrogen and progesterone injections, and exhibited normal mammary ductal development compared with wild-type glands. This suggests that either the presence of the Pgr-B form alone is sufficient to mediate normal ductal development, or it is able to compensate for the lack of Pgr-A. Taken together, these results imply that the particular isoform of Pgr that is expressed, and the relative expression levels of each isoform, are important determinants of Pgr action. To this end, it would be of great interest to investigate whether the expression pattern of the two Pgr isoforms changes significantly over the course of mammary gland development, which would, in turn, illuminate their biological significance.

In the original description of the Prlr mice[22], it was shown that five-week-old virgin mice lacking one ($Prlr^{+/-}$) or both ($Prlr^{-/-}$) *Prlr* alleles had reduced branching and ductal outgrowth compared with mice carrying intact *Prlr* alleles ($Prlr^{+/+}$). Because these mice also suffer from systemic abnormalities, such observations could be a result of either direct or

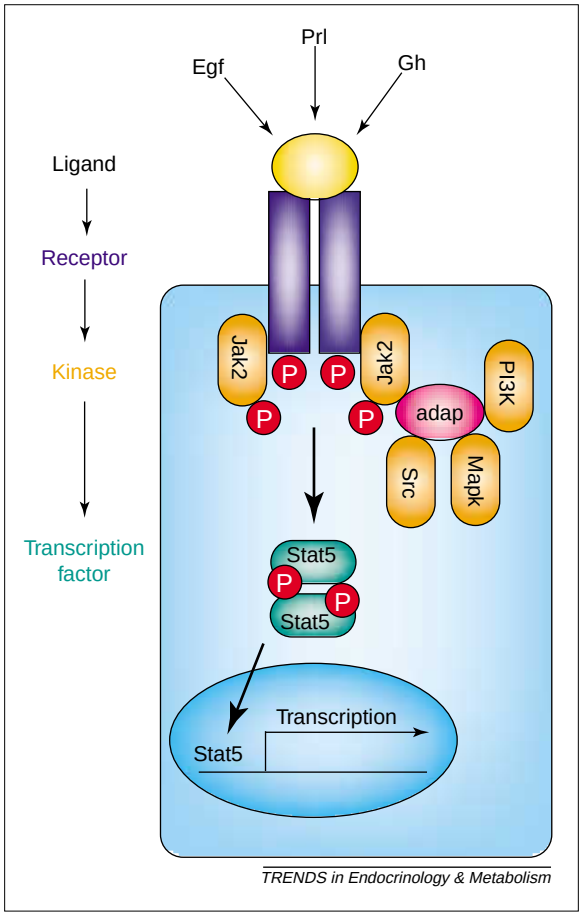

Fig. 3. The Jak–Stat signaling pathway. Ligand binds to its receptor, which then causes dimerization and phosphorylation by either intrinsic kinase activity (Egf) or via a receptor-associated tyrosine kinase, such as Jak2 (Prl and Gh). Phosphorylation of the receptor permits the recruitment of latent Stat transcription factors, in this case Stat5, which are in turn phosphorylated by the kinase. The Stats are then able to dimerize and translocate to the nucleus, where they form large complexes with other transcription factors and coactivators to mediate transcription of specific target genes. Other signaling pathways (PI3K, Src and Mapk) are also activated by receptor-mediated dimerization, most likely via both Jak2-dependent and Jak2-independent mechanisms. Abbreviations: Egf, epidermal growth factor; Jak2, Janus kinase 2; Prl, prolactin; Gh, growth hormone; Stat5, signal transducer and activator of transcription 5; PI3K, phosphatidylinositol-3-kinase; Src, Src kinase; Mapk, mitogen-activated protein kinase; P, phosphorylation; adap, adapter proteins.

indirect mechanisms. To address this issue specifically, transplantation experiments were performed[6,15]. These studies demonstrated that ductal branching and elongation in the mammary transplants during virgin development was normal, suggesting that the phenotype observed in native virgin *Prlr*-null mice was because of either an indirect effect of prolactin on mammary gland development or dysfunctional ovaries. Further analyses of these mice revealed normal development of the mammary gland during pregnancy, but a failure of the gland to establish secretory alveolar structures, demonstrating the importance of the Prlr in lobuloalveolar development. Taken together, these studies define specific and possibly overlapping roles for estrogen, progesterone and prolactin at different times of development.

Although such global analyses have been fundamental in defining a role for these molecules during mammary gland development, they do not address their specific mechanism of action. For example, it is well-established that injection of progesterone leads to proliferation of the ductal epithelium and the appearance of numerous ductal branch points, but until recently, little was known about the cellular events that accompany this observation. Atwood *et al.*[23] have shown that progesterone injection induces Pgr synthesis in structures that resembled developing ductules. Therefore progesterone might regulate branching morphogenesis by upregulating its receptor to induce proliferation. This could potentially be mediated, in part, by the induction of *Wnt4* gene expression, which has been shown to have an essential role in ductal side branching downstream of Pgr (Ref. 24). In addition, ectopic expression of *Wnt1* in *Pgr*-null mice induces ductal side branching, suggesting that the Wnt signaling pathway could play a significant role in progesterone-mediated ductal morphogenesis.

An earlier study[11] established that Tgfb1, which is produced by both stromal and epithelial compartments, also contributes to branching morphogenesis in the virgin mammary gland. Secreted Tgfb1 apparently acts on the extracellular matrix (ECM), and its presence inhibits the formation of ductal branches. Conversely, the notable lack of Tgfb1 is apparent at highly proliferative branch points. In addition to the gene products described above, several other gene products have been shown to play a role in ductal outgrowth and branching morphogenesis[25–27]. This again serves to highlight both the pleiotropic nature of the mammary gland and the importance of cellular interactions within the gland[1,8,28,29].

Stat5, a central transcription factor in mammary gland development

Experiments with genetic knockout models have clearly shown that the Prl–Jak–Stat pathway (Fig. 3) plays a central role in the formation of alveolar structures during pregnancy. The two Stat5 transcription factors, Stat5a and Stat5b, are highly (95%) conserved and differ mainly at their C-termini[30]. Targeted disruption of the *Stat5a* gene has demonstrated that this is the major form of Stat5 controlling mammary gland development and differentiation during pregnancy[31]. *Stat5a*-null mice are unable to lactate owing to reduced epithelial development and a failure of secretory cells to form fully functional secretory alveoli. By contrast, the absence of Stat5b has no overall effect on mammary gland development and *Stat5b*-null mice are able to lactate[32]. The apparent lack of effect of *Stat5b* deletion on mammopoiesis is partly because Stat5b levels in the mammary gland are much lower than those of Stat5a[30,33]. Furthermore, the absence of Stat5b can be completely compensated for by Stat5a[32]. Taken together, these data provide compelling evidence that Stat5a is the dominant Stat5 transcription factor in the mammary gland. However, although these two transcription factors appear to show no apparent overlapping function, Stat5b is able to compensate partially for the lack of Stat5a after multiple pregnancies[34], raising the possibility that Stat5b might play a role during mammary gland development. The generation of Stat5a–Stat5b double-knockout mice[32] will enable us to answer these questions.

Recently, experiments were performed to ascertain whether Stat5 was activated in the stromal compartment, the epithelial compartment, or in both[6]. As suspected from the exclusive synthesis of the Prlr

in the mammary secretory cells, prolactin was shown to activate Stat5 only in the epithelial compartment. By contrast, both growth hormone (Gh) and epidermal growth factor (Egf) preferentially activated Stat5 in the stromal compartment. Further analyses using transplantation demonstrated that the presence of the Prlr in the epithelial compartment is required for mammary gland development and milk-protein expression during pregnancy[15]. However, the absence of the Gh receptor (Ghr) or Egf receptor (Egfr) in the epithelial compartment had no effect on mammary gland development[5], providing evidence for a role confined to the stromal compartment.

New genes on the block

It is now well-established that intracellular signaling via the Pgr, Esr, Ghr, Egfr and Prlr pathways are necessary for morphological and functional development of the mammary epithelium. More recent data have brought to light several novel genes that are intimately involved in mammary gland growth and morphogenesis[35–37].

Receptor activator of NFκB-ligand [RANKL, officially tumor necrosis factor superfamily member 11 (Tnfsf11) and also known as osteoprotegerin-ligand (OPGL), osteoclast differentiation factor (ODF) and Tnf-related activation-induced cytokine (TRANCE)] is a soluble factor produced by osteoclasts, which stimulates the differentiation and activation of osteoclasts involved in bone morphogenesis and remodeling. Deletion of *RANKL* results in severe osteoporosis and a failure of tooth eruption[38]. Interestingly, expression of *RANKL* is regulated by a large variety of hormones[39], including progesterone, estrogen, prolactin and parathyroid hormone-like peptide (Pthlh)[40]. Because the same hormones and growth factors control mammary gland development, this prompted an investigation into whether *RANKL* and its receptor (RANK) might play a role in mammary gland development. Analyses of *RANKL*- and *RANK*-null mice revealed their inability to lactate, and morphological analyses linked this to defects in lobuloalveolar development[35]. Specifically, a significant increase in apoptosis and decreases in proliferation and PKB/Akt activation in developing lobuloalveolar buds were apparent. Implantation of RANKL pellets into pregnant *RANKL*- and *RANK*-null mice restored lobuloalveolar development and PKB/Akt activation in *RANKL*-null mice, but not in *RANK*-null mice. Furthermore, transplantation experiments established that local synthesis of RANKL by the epithelium is necessary for the formation of alveolar buds, because *RANKL*-null epithelium failed to develop in wild-type stroma. This study not only establishes a new signaling axis involved in mammary gland development, but it also highlights the importance of RANKL in the control of mammary epithelial-cell survival and proliferation.

The inhibitor of differentiation and DNA-binding proteins (Idb), Idb1 and Idb2, are dominant–negative helix–loop–helix transcriptional repressors that inhibit the function of basic helix–loop–helix transcription factors[41]. It has been established that the Idb proteins stimulate cell-cycle progression and serve as negative regulators of cell differentiation[42], and Idb1 is involved in the glucocorticoid-mediated regulation of tight junction integrity in the mammary gland[43]. Interestingly, pups delivered by *Idb2*-null female mice rarely survive[36]. Analyses of mammary development in these mice demonstrated a lack of lobuloalveolar development at parturition, which corresponds to a deficiency in functional differentiation. This decrease in lobuloalveolar development was, in part, accounted for by a reduction in Stat5 DNA-binding activity[36]. During early pregnancy (day 7) proliferation is reduced, which coincides with an upregulation of the cyclin-dependent kinase inhibitors, *p21* and *p27*. At mid-pregnancy (day 14), a significant increase in apoptosis is concomitant with an upregulation of *p53*- and *Bax*-expression levels. Based on its known roles, these data suggest that Idb2 might be involved in cell-cycle progression during early pregnancy and cell survival (via a p53–Bax pathway) during mid-pregnancy.

The discoidin domain receptor, Ddr1, is a receptor tyrosine kinase with unknown function that is activated by all collagens tested to date (types I–VI)[44]. *Ddr1* expression occurs in a variety of tissues; most notably, it is expressed in normal mammary epithelium and overexpressed in a number of human breast tumors[45]. To investigate the *in vivo* role of *Ddr1*, a *Ddr1*-null mouse model was generated[37]. Although *Ddr1*-null mice were viable, they were consistently smaller than their wild-type littermates. Consistent with *Ddr1* expression in the uterine wall, most of the *Ddr1*-null females exhibited defects in blastocyst implantation. In spite of this defect, some null mice (20%) were able to successfully carry and deliver their pups but were unable to nurse them owing to a lack of milk secretion. Histological analyses of the mammary epithelium isolated from *Ddr1*-null mice revealed several defects. In three-week-old virgin null mice, ductal outgrowth was retarded and the TEBs were enlarged compared with heterozygous littermate controls. At three months of age there was a considerable increase in the deposition of ECM in the mammary glands of null mice. Furthermore, the ducts were enlarged and hyperproliferative as assessed by Ki-67 staining. At pregnancy day 18.5, when *Ddr* mRNA levels are high, null epithelium failed to completely constitute the fat pad and although milk-protein mRNA could be detected, there was no morphological evidence of milk secretion into the lumen. These observations suggest that *Ddr1* might control mammary epithelial cell morphogenesis and differentiation through two independent mechanisms: suppression of proliferation and deposition of ECM.

Concluding remarks

With conditional targeting approaches, it has been demonstrated that several genes play key roles

throughout the different phases of mammary gland development. Central to the differentiation of mammary epithelium at pregnancy is the Prl–Jak–Stat5 pathway and its associated target genes. However, it is becoming clearer that a large number of genes play a role in mammary gland morphogenesis. To go beyond simple gross observations, it will be necessary to define the defects observed in branching morphogenesis and lobuloalveolar development at a molecular level. This includes the identification of direct target genes activated by the signaling pathways during ductal branching in the virgin gland, lobuloalveolar development during pregnancy and lactation and epithelial regression at involution. Such discoveries will not only add significantly to our current knowledge of normal mammary gland development, but could help us to understand further the development and progression of tumorigenesis.

References

1 Robinson, G.W. *et al.* (1999) Regulation of mammary gland development by tissue interaction. *J. Mam. Gland Biol. Neoplasia* 4, 9–19
2 Hennighausen, L. and Robinson, G.W. (1998) Think globally, act locally: the making of a mouse mammary gland. *Genes Dev.* 12, 449–455
3 Humphreys, R.C. *et al.* (1997) Use of PRKO mice to study the role of progesterone in mammary gland development. *J. Mam. Gland Biol. Neoplasia* 2, 343–354
4 Robinson, G.W. and Hennighausen, L. (1997) Inhibins and activins regulate mammary epithelial cell differentiation through mesenchymal-epithelial interactions. *Development* 124, 2701–2708
5 Wiesen, J.F. *et al.* (1999) Signaling through the stromal epidermal growth factor receptor is necessary for mammary ductal development. *Development* 126, 335–344
6 Gallego, M.I. *et al.* (2001) Prolactin, growth hormone, and epidermal growth factor activate Stat5 in different compartments of mammary tissue and exert different and overlapping developmental effects. *Dev. Biol.* 229, 163–175
7 DeOme, K.B. *et al.* (1959) Development of mammary tumors from hyperplastic alveolar nodules trasnplanted into gland-free mammary fat pads of female C3H mice. *Cancer Res.* 19, 515–520
8 Cunha, G.R. *et al.* (1997) Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J. Mam. Gland Biol. Neoplasia* 2, 393–402
9 Humphreys, R.C. *et al.* (1997) Mammary gland development is mediated by both stromal and epithelial progesterone receptors. *Mol. Endocrinol.* 11, 801–811
10 Silberstein, G.B. (2001) Role of the stroma in mammary development. *Breast Cancer Res.* 3, 218–223
11 Silberstein, G.B. *et al.* (1992) Regulation of mammary morphogenesis: evidence for extracellular matrix-mediated inhibition of ductal budding by transforming growth factor-beta 1. *Dev. Biol.* 152, 354–362
12 Gouon-Evans, V. *et al.* (2000) Postnatal mammary gland development requires macrophages and eosinophils. *Development* 127, 2269–2282
13 Korach, K.S. *et al.* (1996) Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Rec. Progr. Horm. Res.* 51, 159–186
14 Brisken, C. *et al.* (1998) A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc. Natl. Acad. Sci. U. S. A.* 95, 5076–5081
15 Brisken, C. *et al.* (1999) Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev. Biol.* 210, 96–106
16 Lydon, J.P. *et al.* (1995) Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev.* 9, 2266–2278
17 Ormandy, C.J. *et al.* (1997) Mammary gland development in prolactin receptor knockout mice. *J. Mam. Gland Biol. Neoplasia* 2, 355–364
18 Krege, J.H. *et al.* (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proc. Natl. Acad. Sci. U. S. A.* 95, 15677–15682
19 Mulac-Jericevic, B. *et al.* (2000) Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 289, 1751–1754
20 Bocchinfuso, W.P. *et al.* (2000) Induction of mammary gland development in estrogen receptor-alpha knockout mice. *Endocrinology* 141, 2982–2994
21 Shyamala, G. *et al.* (1998) Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. *Proc. Natl. Acad. Sci. U. S. A.* 95, 696–701
22 Ormandy, C.J. *et al.* (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.* 11, 167–178
23 Atwood, C.S. *et al.* (2000) Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice. *J. Endocrinol.* 167, 39–52
24 Brisken, C. *et al.* (2000) Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev.* 14, 650–654
25 Ruan, W. and Kleinberg, D.L. (1999) Insulin-like growth factor I is essential for terminal end bud formation and ductal morphogenesis during mammary development. *Endocrinology* 140, 5075–5081
26 Crowley, M.R. *et al.* (2000) The mouse mammary gland requires the actin-binding protein gelsolin for proper ductal morphogenesis. *Dev. Biol.* 225, 407–423
27 Ilan, N. *et al.* (2001) Pecam-1 is a modulator of stat family member phosphorylation and localization: lessons from a transgenic mouse. *Dev. Biol.* 232, 219–232
28 Zangani, D. *et al.* (1999) Multiple differentiation pathways of rat mammary stromal cells *in vitro*: acquisition of a fibroblast, adipocyte or endothelial phenotype is dependent on hormonal and extracellular matrix stimulation. *Differentiation* 64, 91–101
29 Darcy, K.M. *et al.* (2000) Mammary fibroblasts stimulate growth, alveolar morphogenesis, and functional differentiation of normal rat mammary epithelial cells. *In vitro Cell Dev. Biol. Anim.* 36, 578–592
30 Liu, X. *et al.* (1995) Cloning and expression of Stat5 and an additional homologue (Stat5b) involved in prolactin signal transduction in mouse mammary tissue. *Proc. Natl. Acad. Sci. U .S. A.* 92, 8831–8835
31 Liu, X. *et al.* (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.* 11, 179–186
32 Teglund, S. *et al.* (1998) Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 93, 841–850
33 Liu, X. *et al.* (1996) Activation of Stat5a and Stat5b by tyrosine phosphorylation is tightly linked to mammary gland differentiation. *Mol. Endocrinol.* 10, 1496–1506
34 Liu, X. *et al.* (1998) Functional release of Stat5a-null mammary tissue through the activation of compensating signals including Stat5b. *Cell Growth Differ.* 9, 795–803
35 Fata, J.E. *et al.* (2000) The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 103, 41–50
36 Mori, S. *et al.* (2000) Lactation defect in mice lacking the helix–loop–helix inhibitor Idb2. *EMBO J.* 19, 5772–5781
37 Vogel, W.F. *et al.* (2001) Discoidin domain receptor 1 tyrosine kinase has an essential role in mammary gland development. *Mol. Cell. Biol.* 21, 2906–2917
38 Kong, Y.Y. *et al.* (1999) OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature* 397, 315–323
39 Ross, F.P. (2000) RANKing the importance of measles virus in Paget's disease. *J. Clin. Invest.* 105, 555–558
40 Karsenty, G. (1999) The genetic transformation of bone biology. *Genes Dev.* 13, 3037–3051
41 Benezra, R. *et al.* (1990) Idb: a negative regulator of helix–loop–helix DNA binding proteins. Control of terminal myogenic differentiation. *Ann. New York Acad. Sci.* 599, 1–11
42 Norton, J.D. (2000) ID helix–loop–helix proteins in cell growth, differentiation and tumorigenesis. *J. Cell Sci.* 113, 3897–3905
43 Woo, P.L. *et al.* (2000) Involvement of the helix–loop–helix protein Idb-1 in the glucocorticoid regulation of tight junctions in mammary epithelial cells. *J. Biol. Chem.* 275, 28649–28658
44 Vogel, W. *et al.* (1997) The discoidin domain receptor tyrosine kinases are activated by collagen. *Mol. Cell* 1, 13–23
45 Barker, K.T. *et al.* (1995) Expression patterns of the novel receptor-like tyrosine kinase, Ddr, in human breast tumours. *Oncogene* 10, 569–575
46 Chapman, R.S. *et al.* (1999) Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev.* 13, 2604–2616
47 Seagroves, T.N. *et al.* (1998) C/EBPbeta, but not C/EBPalpha, is essential for ductal morphogenesis, lobuloalveolar proliferation, and

functional differentiation in the mouse mammary gland. *Genes Dev.* 12, 1917–1928
48 Robinson, G.W. *et al.* (1998) The C/EBPbeta transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev.* 12, 1907–1916
49 Seagroves, T.N. *et al.* (2000) C/EBPbeta (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. *Mol. Endocrinol.* 14, 359–368
50 Sebastian, J. *et al.* (1998) Activation and function of the epidermal growth factor receptor and erbB-2 during mammary gland morphogenesis. *Cell Growth Differ.* 9, 777–785
51 Luetteke, N.C. *et al.* (1999) Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126, 2739–2750
52 Kloetzli, J.M. *et al.* (2001) The winged helix gene, Foxb1, controls development of mammary glands and regions of the CNS that regulate the milk-ejection reflex. *Genesis* 29, 60–71
53 Gorska, A.E. *et al.* (1998) Dominant-negative interference of the transforming growth factor beta type II receptor in mammary gland epithelium results in alveolar hyperplasia and differentiation in virgin mice. *Cell Growth Differ.* 9, 229–238
54 Toscani, A. *et al.* (1997) Arrest of spermatogenesis and defective breast development in mice lacking A-myb. *Nature* 386, 713–717
55 Zhou, Y. *et al.* (1997) A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proc. Natl. Acad. Sci. U. S. A.* 94, 13215–13220
56 Horseman, N.D. *et al.* (1997) Defective mammopoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J.* 16, 6926–6935
57 Pollard, J.W. and Hennighausen, L. (1994) Colony stimulating factor 1 is required for mammary gland development during pregnancy. *Proc. Natl. Acad. Sci. U. S. A.* 91, 9312–9316
58 Young, W.S., III *et al.* (1996) Deficiency in mouse oxytocin prevents milk ejection, but not fertility or parturition. *J. Neuroendocrinol.* 8, 847–853
59 Wagner, K.U. *et al.* (1997) Oxytocin and milk removal are required for post-partum mammary-gland development. *Genes Funct.* 1, 233–244
60 Nagato *et al.* (1980) Scanning electron micrograph study of myoepithelial cells in exocrine glands. *Cell Tissue Res.* 209, 1–10

Clinical use of a growth hormone receptor antagonist in the treatment of acromegaly

William M. Drake, Craig Parkinson, G. Michael Besser and Peter J. Trainer

The elucidation of the mechanisms by which growth hormone (GH) interacts with its receptor has facilitated the design of compounds that function as GH-receptor antagonists. One such compound, B2036, has been conjugated to polyethylene glycol to produce a drug, pegvisomant, that has a powerful ability to lower circulating concentrations of insulin-like growth factor I (IGF-I), the principal mediator of GH action, in patients with acromegaly and to improve the symptoms and signs associated with GH excess. This article describes the mechanism of action of GH-receptor antagonists, reviews the preclinical and clinical data on the use of pegvisomant and discusses some of the challenges that lie ahead in judging the efficacy of a treatment that, unlike established therapies for acromegaly, does not aim to modify the underlying cause of acromegaly, namely excess GH secretion, but aims to lower serum IGF-I levels to normal.

William M. Drake
G. Michael Besser
Dept Endocrinology, St Bartholomew's Hospital, London, UK EC1A 7BE.

Craig Parkinson
Peter J. Trainer*
Dept Endocrinology, Christie Hospital, Manchester, UK M20 4BX.
*e-mail: peter.trainer@man.ac.uk

Growth hormone (GH) is a 191 single-chain polypeptide synthesized and secreted by somatotroph cells of the anterior pituitary. In the circulation, GH is bound to two separate GH-binding proteins (GHBPs), one of lower affinity[1] and one – the extracellular portion of the GH receptor – of higher affinity[2]. Although GH probably has some direct effects on peripheral tissues, such as epiphyseal chondrocytes[3], the majority of its actions are mediated through the peptide hormone insulin-like growth factor-I (IGF-I), a member of the insulin-like peptide family. Transcriptional activation of the gene encoding IGF-I, leading to IGF-I synthesis and secretion, occurs in response to a GH-mediated signal. X-ray crystallography[4] and Ala-scanning mutagenesis[5] have shown that this GH signal is mediated through a complex that includes a single GH molecule and two identical GH cell-surface receptors[6]. The GH molecule contains two separate receptor-binding domains: site 1 and site 2 (Ref. 7). The secondary structure of GH consists of a four α-helix core with two disulfide bonds, such that noncontiguous regions of the amino acid chain contribute to the two binding regions. Site 1 is made up of the loop between amino acid residues 54 and 74, the C-terminal half of helix four and the *N*-terminal region of helix one. The *N*-terminal residues of the first and third helices contribute to binding-site 2 (Ref. 7). Once GH binds to an initial GH receptor via site 1, a second, identical, receptor is recruited, leading to receptor dimerization and subsequent cellular activation.

GH-receptor antagonists

Before the elucidation of the precise mechanisms of GH signaling via its receptor, it had been demonstrated that expression of a mutant GH molecule suppresses the growth of transgenic mice[8]. Subsequently, it was shown that the important amino acid residues of the GH molecule that produce this effect are in the third helix, in the region of site-2 binding[9]. Mutations that result in substitution of any amino acid (other than Ala) for Gly at position 120 (in the region of site-2 binding) sterically inhibit GH binding to its receptor. When mutations in the region of site-2 binding are combined with amino acid substitutions in the region of binding site-1 that result in an increased binding

 1043-2760/01/$ – see front matter PII: S1043-2760(01)00461-1